International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-007, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Highly virulent *Photobacterium damselae* subsp. *piscicida* isolated from Taiwan paradise fish, *Macropodus opercularis* (L.), in Taiwan

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Accepted 15 January, 2019

In Taiwan, a fish conservation farm had about 6,250 Taiwan paradise fishes, *Macropodus opercularis* (L.), which were separately cultivated in an outdoor tank containing 3,250 fishes, and an indoor tank containing the rest. The water supplying both tanks was the same running water source from the adjacent hills. Following a change in the fish feed (the commercial aquaculture feed became eel's feed) to all fishes in May 2010, rotten body surfaces were only found in diseased and dead fishes in the outdoor tank. Interestingly, no sick fishes were found in the indoor tank. The clinical findings of the diseased fishes were bleeding at basal fins, peripheral site of genital pore, and bilateral surface of the abdomen. Additionally, we discovered whitish-mucus gills, edema of the intestines, and multi-focal white tubercles in infected fishes during gross examination. The results of the histopathology study showed that there were numerous multi-focal granulomas in the spleen, posterior kidney, and liver. Furthermore, *Photobacterium damselae* subsp. *piscicida* was isolated from lesions of the ailing fishes. We conducted an experimental animal virulence test, and our data revealed that *P. damselae* subsp. *piscicida* was a highly virulent pathogen. Fortunately, *P. damselae* subsp. *piscicida* appeared to be susceptible to most commonly used antimicrobial agents, according to the results of the antibiotic sensitivity study. We recommended a treatment with oxolinic acid (20 mg/kg/day) in the feed for 7 days for all Taiwan paradise fishes. The fishes' condition significantly improved and the disease appeared to be controlled.

Key words: Conservation, photobacteriosis, Taiwan paradise fish, virulence.

INTRODUCTION

The Taiwan paradise fish, *Macropodus opercularis* (L.), is evenly dispersed on the plains or lower- elevation hills of western and northeastern Taiwan, but its population has fluctuated considerably in recent years. According to several studies (Shen et al., 1991; Jan and Wu, 1994), there still remain scattered populations of *M. opercularis* (L.) in western Taiwan. The fish prefers to live in natural ponds or minor creeks. The broad distribution has dwindled to a sporadic occurrence according to several investigations (Shen et al., 1991; Jan and Wu, 1994). Therefore, the Taiwan government placed the Taiwan paradise fish on the rare and valuable species list on 31 August. 1990. *M. opercularis* (L.) is a very fastidious fish

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Abbreviations: TSA, tryptic soy agar; **i.p.,** intraperitoneal.

which does not tolerate changes in environmental conditions. Hence, this fish is used as an environmental pollution index for monitoring of environmental pollution in Taiwan. In recent years, drastic environmental changes have led to a severe reduction in the genetic diversity and survival rate of this fish population. Therefore, appropriate artificial breeding is necessary to gradually increase its numbers in the wild (Jan and Wu, 1994). There are several private fish farms in Taiwan which have been set up for this purpose.

Pasteurellosis or pseudotuberculosis, also currently described as photobacteriosis, is infected by the halophilic bacterium *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), which was initially isolated from white perch and striped bass in the Chesapeake Bay, USA in 1963 (Snieszko et al., 1964). In Europe and the Mediterranean, photobacteriosis was first isolated from the juvenile gilthead sea bream (*Sparus aurata*) in the northwest of Spain in 1991 (Toranzo et al., 1991).

Hawke (1996) also reported a significant monetary loss, about US$ 1 million, from the loss of valuable food-size fishes due to photobacteriosis at a Louisiana fish farm from 1991 to 1994. In Japan, photobacteriosis is considered a major disease which can cause economic losses in cultured fishes such as yellowtail juveniles (*Seriola quinqueradiata*) (Kubota et al., 1970; Kusuda and Yamaoka, 1972), ayu (*Plecoglossus altivelis*) (Kusuda and Miura, 1972), black sea bream (*Mylio macrocephalus*) (Muroga et al., 1977; Ohnishi et al., 1982), red sea bream (*Acanthopagrus schlegeli*) (Yasunaga et al., 1983), oval file fish (*Navodan modestus*) (Yasunaga et al., 1984), and red grouper (*Epinephelus okaara*) (Ueki et al., 1990). Moreover, this disease has been reported in the snake-head fish (*Channa maculata*) in Taiwan (Tung et al., 1985) . This disease seems to be a threat to all cultured farms in the world. Therefore, the prevention and control of this disease has become a very important issue. In this study we found that photobacteriosis was able to cause serious infection, even death, in the Taiwan paradise fish, *M. opercularis* (L.). As such, signs of infection with photobacteriosis should be not ignored.

MATERIALS AND METHODS

Bacterial isolation

P. damselae subsp. *piscicida* was isolated from posterior kidney, spleen, skin, and liver from sick Taiwan paradise fishes, *M. opercularis* (L.) using tryptic soy agar (TSA) plates (Difco) with 5% sheep blood. Fish age is between 3-6 month old, body weight is 0.2-0.6 g, and water temperature of culture at 28°C. The isolated strain was characterized and identified according to standard morphological, physiological, and biochemical techniques (Gauthier et al., 1995; Truper and Declari, 1997; Abbasi et al., 2010). Each test was performed in triplicate at 25°C for 48 h. At the same time, all test results were compared with those of a standard strain, *P. damselae* subsp. *piscicida* ATCC 51736 (Gauthier et al., 1995; Truper and Declari, 1997).

Sensitivity of *P. damselae* subsp. *piscicida* to various antimicrobic agents

P. damselae subsp. *piscicida* was grown on TSA at 25°C for 24 h. Then the bacteria were suspended in sterile phosphate buffered saline [PBS: 0.8% (w/v) NaCl, 0.02% (w/v) KCl, 0.02% (w/v) KH_2PO_4, 0.1 % (w/v) Na_2HPO_4, 10% (v/v) glycerol; pH 7.2] and diluted as the MacFarland No. 0.5 standard solution tube (0.5 mL $BaSO_4$ + 99.5 mL 0.36 N HCl), about 1×10^7 CFU/mL. The bacterial diluted suspension (0.1 mL) was spread onto a Mueller-Hinton agar (Difco) then selected antibiotic discs were added on it (Koneman et al., 1988). The antibiotic discs used in this assay included getamicin (10 g), oxolinic acid (2 g), oxytetracycline (30 g), SXT-TS1 (23.75 g sulfamethoxazole with 1.25 g trimethoprim), and ampicillin (10 g). All tested plates were incubated at 25°C for 18 h. After the above procedures, the bacterial inhibition zone of each tested antibiotic agent was noted. The antibiotic sensitivities of our isolated bacterium were then interpreted and recorded (Koneman et al., 1988).

Fish and virulence tests

There are five groups in this study. Each of the five Taiwan paradise fishes in each test group received a different amount of bacterial suspension, including 1.2×10^1 CFU/mL, 1.2×10^2 CFU/mL, 1.2×10^3 CFU/mL, and 1.2×10^4 CFU/mL/fish of about 1 g and 5 cm in body weight and length of *M. opercukaris* (L.), were respectively held in a tank (100 liter) for testing. Each fish in each tested group received intraperitoneal (i.p.) injections with different amounts of bacterial suspension (0.1 mL/fish) (Trevors and Lusty, 1985) . Fishes inoculated with sterile PBS by i.p. served as the parallel control. Mortalities were monitored and recorded daily for 7 days after the shots. Re-isolation and identification of the bacteria from posterior kidney and liver of moribund Taiwan paradise fishes was also performed.

Characteristics and identification of *P. damselae* subsp. *Piscicida*

Morphological and biochemical characteristics of our isolate were performed as previous description (Gauthier et al., 1995). These examined items included morphological appearance, bacterial growth, and biochemical characteristics as enzyme activity and utilization of carbohydrates (Table 1).

RESULTS

Characteristics and identification of *P. damselae* subsp. *piscicida*

We isolated a uniquely pure colony from ill fish organs. The staining characteristics of our pleomorphic rod-shape isolate were Gram-negative with bipolar staining and were bioluminescent-negative. Its size was about 0.5-0.8 by 0.7-2.6 m. The bacterium did not have flagella; therefore, its motility could not be observed. The biochemical characteristics of our strain were β-hemolysis activity, facultative anaerobic activity, and non-gas producing sugar fermentation/oxidation, but there was inactivity of nitrate reductase, lipase, gelatinase, esculinase and urease. Additionally, there was phospholipase activity in

Table 1. Biochemical characteristics of *P. damselae* subsp*. piscicida* compared with the standard strain *P. damselae* subsp*. piscicida* ATCC 51736.

Biochemical characters	*P. damselae* subsp*. piscicida*	ATCC 51736
Gram stain	-	-
Motility	-	-
Hemolysis	β	β
Bioluminescence	-	-
Growth at:		
4°C	-	-
35°C	+	+
Nitrate reductase	-	-
Oxidase activity	+	+
Catalase activity	+	+
Urease activity	-	-
Indole	-	-
MR test	+	+
Utilization of:		
Gluconate	-	-
Maltose	-	-
D-Xylose	-	-
Cellobiose	-	-
Glutamate	+	+
Acetate	-	-
Pyruvate	-	-

'+' indicates positive, while '-' indicates negative

P. damselae subsp. *piscicida* (Table 1).

Furthermore, the results of oxidase, catalase, L-proline, adipate, glutamate, and methyl red tests were positive, but negative for indole, H_2S production tests, and utilization of D- xylose, maltose, cellobiose, glutamate, acetate, pyruvate. Based on the above test results, our isolate was identified as *P. damselae* subsp. *piscicida* as previous description (Gauthier et al., 1995).

Gross examination and histopathologic study

Diseased fishes were lethargic and tended to sink while swimming. Pigment formation was not well regulated, and respiration rate was increased (Figure 1A). The gross examination revealed petechiae in the opercular region, at the base of the fins, and inside the mouth in ill fishes. Pallor of the gills of sick fishes was also found. The histopathologic study revealed many slightly mottled and whitish spots which were identified as 1 to 2 mm granuloma in the livers of the ailing fishes (Figure 1B).

Moreover, the same lesions were observed in the swollen spleen, liver, and kidney of diseased fishes (Figure 1B). Although the lesions grossly resembled true granulomas in the liver, the injury was categorized as "pseudotuberculosis." In our study, there were many granulomas located in the liver (Figure 2A), posterior kidney (Figure 2B), and muscle (Figure 2C). Furthermore, we found multi-focal areas of renal tubule undergoing coagulation necrosis in the posterior kidney of the infected fishes (Figure 2D).

Sensitivity of *P. damselae* subsp. *piscicida* to various antimicrobic agents

Five antimicrobial agents were used in this study, including getamicin, oxolinic acid, oxytetracycline, SXT-TS1 (sulfamethoxazole with trimethoprim), and ampicillin for antibiotics sensitivity test. Our results demonstrated that *P. damselae* subsp. *piscicida* was sensitive to getamicin, oxolinic acid, oxytetracycline, and SXT-TS1, but not ampicillin (Table 2).

Virulence tests of *P. damselae* subsp. *piscicida*

From the results of the virulence tests, *P. damselae* subsp. *piscicida* was found to possess high virulence. A dose of 1.2×10^{1} CFU/mL of *P. damselae* subsp. *piscicida* was able to kill all fishes in 4 days. Higher doses of *P. damselae* subsp. *piscicida* killed the fishes in 2 days (Table 3). (This is hard to understand based on the virulence test. Say how many groups, how many fish in

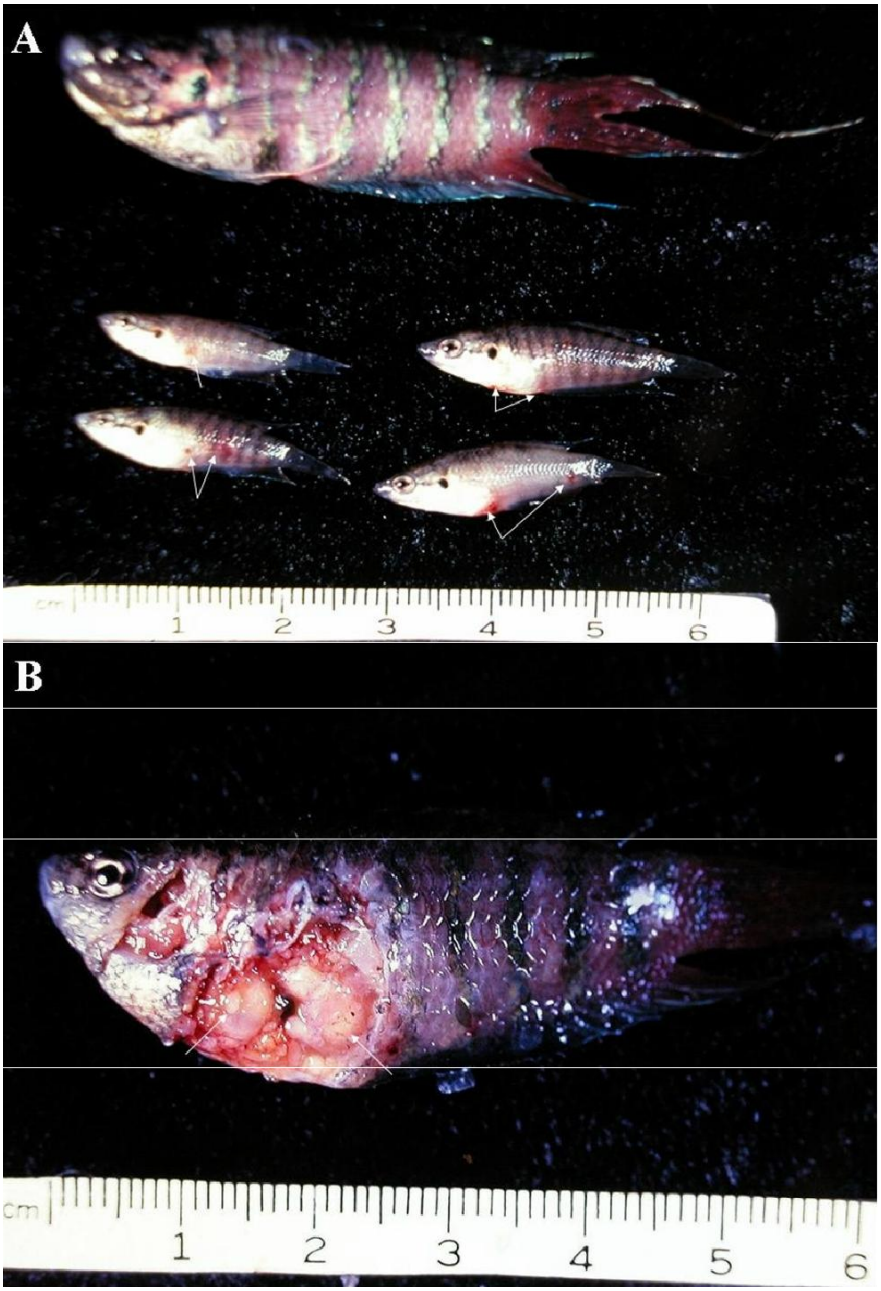

Figure 1. Gross findings of diseased Taiwan paradise fish, *M. opercularis* (L.). (A) Hemorrhagic spots were noted on the skin surface of diseased Taiwan paradise fish (arrows); (B) Diseased Taiwan paradise fish revealed white nodules on visceral organs (arrows).

each group, and what you did to each group) The clinical findings, gross lesions, and histopathological changes of the challenged-moribund fishes in the virulence test were the same as those found in a natural outbreak. A homogenous colony bacterium was re-isolated from the kidney and liver of moribund injected fishes. The pure cultured isolate was identified as the same as that of the diseased fishes. No dead fishes were found in the control group which comprised fishes injected with sterile PBS.

DISCUSSION

Environmental pollution is a very serious problem in Taiwan and it is having a devastating effect on numerous species of wild fish and animals. The paradise fish, *M.*

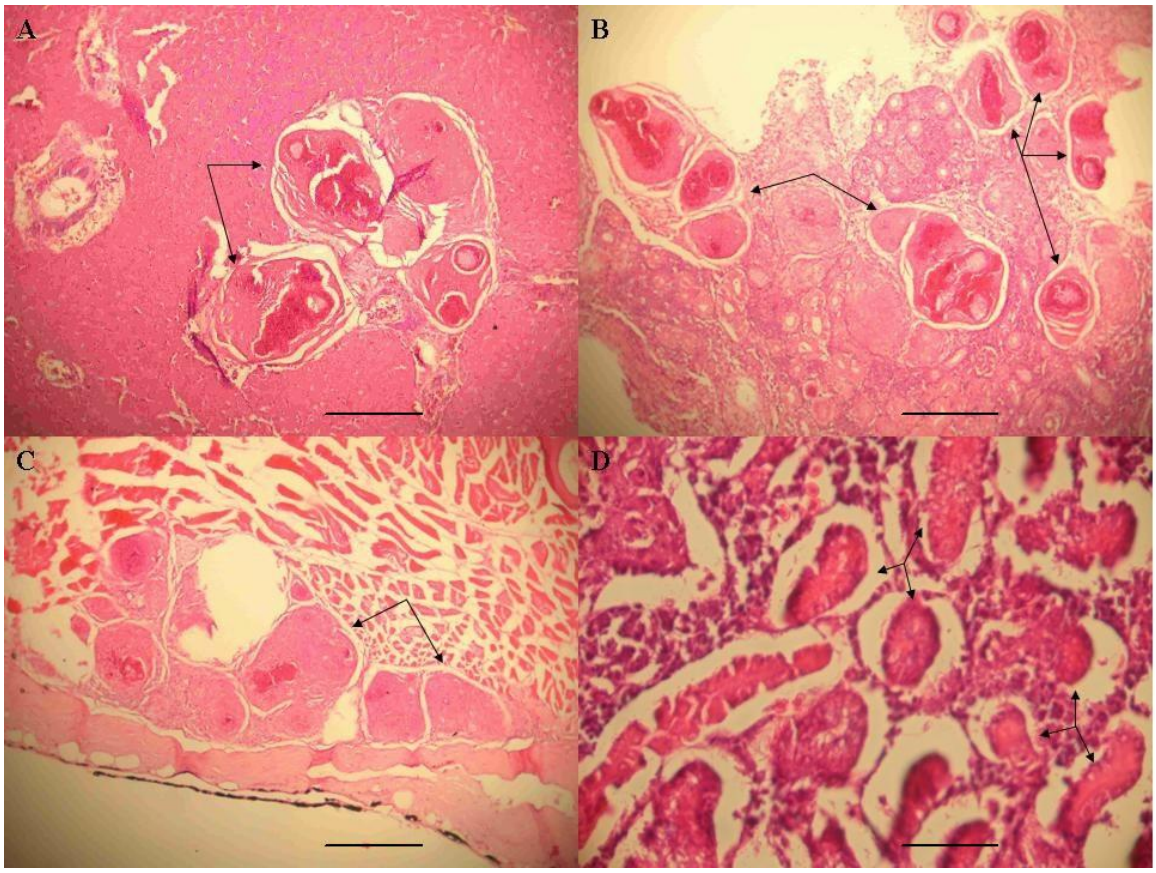

Figure 2. The histopathologic lesions of diseased Taiwan paradise fish, *M. opercularis* (L.) by H and E stain. (A) Liver granulomas (arrows) (B) Posterior kidney granulomas (arrows) (C) Granulomas were evident in muscle (arrows) (D) Multi-focal coagulative necrosis were present in posterior kidney tubules (arrows). Scale bar = 20 μm.

Table 2. Antimicrobial agent sensitivities of *P. damselae* subsp. *Piscicida*.

Antibiotic	mm	interpretation
Gentamicin	24	S
Oxolinic acid	40	S
Oxytetracycline	39	S
SXT–TS1	42	S
Ampicillin	0	R

a. The quantity of drug disc: gentamicin (10 μg), oxolinic acid (2 μg), oxytetracycline (30 μg), SXT–TS1 (23.75 g + 1.25 μg), and ampicillin (10 μg).
b. The inhibitory zones (mm) of gentamicin, oxolinic acid, oxytetracycline, SXT–TS1, and ampicillin were interpreted as being sensitive at lengths exceeding 15, 20, 19, 28, and 17 mm, respectively.
c. 'S' indicates susceptibility to antibiotics, while 'R' indicates resistance. d. SXT–TS1 is sulfamethoxazole added with trimethoprim.

opercukaris (L.) is very sensitive to changes in its environment so it is used as a pollution index in Taiwan. Because of ecological contamination, wild populations of paradise fish have dwindled to dangerous levels since 1991 (Shen et al., 1991; Jan and Wu, 1994). A concerted effort has been made to conserve this fish in the wild and increase its population. However, enhancement of cultured water quality and prevention of contamination by pathogens in cultured ponds have posed major challenges in the conservation of this species.

The conditions favoring the establishment of photobacteriosis by this highly pathogenic halophilic organism have been created due to the development of intensive aquaculture in the United States, Japan, Europe, and the Mediterranean region. This pathogen has proven to be detrimental to wild and farmed fishes, and is responsible for severe losses of cultured yellowtail juveniles (Kubota et al., 1970; Kusuda and Yamaoka, 1972), and mortalities in other cultured fishes, including Ayu, Red sea bream, Black sea bream, Oval file fish, and Red grouper in Japan (Acosta et al., 2006; do Vale et al., 2005). Before 1990, there were no reported cases of photobacteriosis in Europe. Subsequently, epizootics were reported in many areas in Europe, beginning with a gilthead sea bream outbreak in northwestern Spain in 1990 (Toranzo et al., 1991). Simultaneously, epizootics in sea bass occurred in France, Turkey, and Greece. At almost the same time, cases of infection were found in gilthead sea bream in Italy, Malta, and Portugal. In our study, 3,250 Taiwan

Table 3. Taiwan paradise fish, *M. opercularis* (L.) infected with *P. damselae* subsp*. piscicida* via intraperitoneal injection.

Group	Fish No.	Injected bacterial amount (CFU/mL)	No. of dead fish after injection with *P. damselae* subsp*. Piscicida* day						
			1st	2nd	3rd	4th	5th	6th	7th
A	5	1.2×10^{4}	3	2	-	-	-	-	-
B	5	1.2×10^{3}	1	4	-	-	-	-	-
C	5	1.2×10^{2}	1	2	2	0/	-	-	-
D	5	1.2×10^{1}	0	2	2	1	-	-	-
E	5	PBS	0	0	0	0	0	0	0

Groups A to D were injected with *P. damselae* subsp*. piscicida* but group E was injected with 0.1 ml sterile PBS (pH 7.4).

paradise fish died by photobacteriosis only in outdoor tank and the mortality is 100% (3,250/3,250). Consequently, this disease has seriously hindered conservation of the paradise fish, *M. opercukaris* (L.) in Taiwan.

Hawke et al. (1996) reported that photobacteriosis epizootics in cultured hybrid striped bass generally resulted in mortality rates of about 5 to 90% depending on the water quality and medical treatment. They found that this disease usually occurred in the spring or fall. Spring epizootics were characterized by sudden onset of mortality and a very steep mortality curve that was able to reach 80% in 10 days. Interestingly, they indicated that the period of mortalities in fall outbreaks were more prolonged. The reason for that is still unknown. The rapid onset of mortality with little warning allows an infection to become established in cultured fishes before therapy can be initiated (Hawke, 1996).

Despite of a change in the fish feed (the commercial aquaculture feed became eel's feed) in the all tanks, mortalities were found only in the outdoor tank in this case, but not in the indoor one. On the other hand, water temperature is an important point in photo- bacteriosis outbreaks (Hawke, 1996). In this case, we observed different water temperature in the outdoor tank during the night, however, the differences of water temperature was not found in the indoor tank. Therefore, the temperature drops might be the major reason which may have decreased the fishes' immunity. We found many granulomas located in the liver, posterior kidney, and muscle of infected fishes. These may have played a role in weakening and killing the fishes. In addition, multi-focal areas of renal tubule undergoing coagulation necrosis were seen in the posterior kidney of the diseased fishes. It is possible that these resulted in anemia or decreased immunity. Opportunistic pathogens may have then invaded the immunocompromised fishes.

Liu et al. (2003) pointed out that *P. damselae* subsp. *piscicida* can induce death in cobia, *Rachycentron canadum*. The LD_{50} values of *P. damselae* subsp. *piscicida* for cobia was 1.03×10^{4} CFU/mL. In our results, all fishes were dead 4 days after injection with 1.2×10^{1} CFU/mL of *P. damselae* subsp. *piscicida* via i.p. Nevertheless, after injection with 1.2×10^{3} and 1.2×10^{4} CFU/mL, all fishes died within 2 days. Magariños et al. (1994) also demonstrated that *P. damselae* subsp. *piscicida* was highly pathogenic, not only for its infected host fish but also for other fish species. They also reported that the LD_{50} values of the bacterium ranged from 10^{3} to 10^{6} CFU per fish. Therefore, the degree of virulence was related with host and bacterium. In our case, *P. damselae* subsp. *piscicida* seemed to possess a viru-lent capable of inducing high mortality in Taiwan paradise fish.

Kim et al. (2005) showed *P. damselae* subsp. *piscicida* was able to acquire quinolone-resistance genes. Liu et al. (2003) demonstrated that their *P. damselae* subsp. *piscicida* strain was sensitive to ciprofloxacin, erythro-mycin, kanamycin, neomycin, nitrofurantoin, novobiocin, OA, and streptomycin, but not ampicillin, chloramphenicol, doxycyclin, furazolidone, nalidixic acid, oxytetracycline, penicillin G, sulphonamides, tetracycline, or vancomycin. In the present study, our results revealed that the *P. damselae* subsp. *piscicida* isolate was sensitive to oxytetracycline, OA, gentamicin, and SXT-TS1. The main reason for the differences in sensitivity between our study and those of others may be that rare anti-microbial agents were used for preventing diseases in this farm. Another possible reason is that running water was used for the fishes in our study. This reduces the chance of prolonged contact with pathogens which may promote development of resistant genes.

Based on previously articles (Hussain et al., 1990; Acosta et al., 2006) and our experience of treating this disease we recommend that outbreaks of photobac-teriosis in paradise fish be managed with OA (20 mg/kg/day for 7 days). Treatment must be accompanied by a strategy to improve water quality management.

Conflict of interest statement

No potential conflicts of interest were disclosed.

ACKNOWLEDGEMENTS

We would like to thank the Council of Agriculture in Taiwan (Executive Yuan) for supporting this study (98AS-9.2.4-BQ-B1(21) and 99AS-9.2.2-BQ-B1(28)).

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